Report on Mammalian Chromosome Aberration Test for BS 1701

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by Chemical Evaluation and Research Institute, Japan

Summary

Using lung fibroblasts of Chinese hamsters (CHL/IU cells) we examined the possibility of inducing x-chromosomal aberrations.

In the absence of S9 mix doses were adjusted to 22, 28 and 34 μg/ml and in the presence of S9 mix to 690, 1,380 and 2,760 μg/ml. The chromosomal aberration experiments using short-time processing revealed that in the presence of S9 mix BS1701 did not increase the incidence of x-chromosomal aberrations, while in the absence of S9 mix 28-34 μg/ml induced an increase in structural anomalies.

The above results suggest that under the present experimental conditions BS1701 induces chromosomal aberrations.

Test materials and testing method

1. Samples and positive reference

1.1 Samples provided by client

1) Name
A mixture of 2,4,4-trimethylpentyltriethoxysilane and 5,5-dimethylhexyltriethoxysilane

other name: BS1701

CAS No: 35435-21-3(2,4,4-trimethylpentyltriethoxysilane)

2) Lot No

KH02168

3) Sample provider

Asahikasei Wacker Silicone Co., Ltd.

4) Chemical structure

A(2,4,4-trimethylpentyltriethoxysilane)

B(5,5-dimethylhexyltriethoxysilane)

A: 91 w/w% B: 6 w/w%

5) Purity

more than 97w/w%

6) Impurities and those content

Ethanol less than 1%, Silicone(disiloxane derived from A and B) less than 1%

7) Physical and chemical properties

At ambient temperature	transparent liquid
Molecular weight	276.49
Stability	stable at ambient temperature
Melting point	-
Boiling point	112 degree C(14 hPa)
Vapor pressure	<40 hPa(20 degree C), <100 hPa(50 degree C)
Distribution Coeffcient	-
Density	0.86 g/cu.cm
Hydrolysis	yes
Solubility	
Water	-
DMSO	less than 50 mg/ml(measure at the laboratory)
acetone	more than 276 mg/ml(measure at the laboratory)
others	-

1.2 Positive control substances

1) Mitomycin C (MMC)

Manufacturer: Kyowa Hakko Kogyo
Lot No.: 284AIG
Appearance: blue-violet crystals or crystalline powder
Composition: 1 vial contains 2 mg of Japanese Pharmacopoeia grade mitomycin C (potency) and 48 mg of Japanese Pharmacopoeia grade sodium chloride.
Grade: for injection

2) Cyclophosphamide hydrate (CPA)

Manufacturer: Wako Pure Pharmaceuticals Inc.
Lot No.: LEE7264
Appearance: white crystalline powder
Purity: 98.9%
Grade: biochemical

3) Storage conditions

Store MMC at room temperature and CPA in a cool, dark place.

4) Handling precautions

Wear gloves, mask, head cover and white gown.

2. Cells and culture media used

2.1 Cells and reasons for their selection

Chinese hamster lung fibroblasts (CHL/IU cells) used for experiments: the clone number 11) had been distributed by the National Institute of Hygienic Sciences (currently National Drug and Food Laboratory) on September 28 1988 and were the 18th subculture generation at the time of distribution. We confirmed in our facility that the number of mode chromosomes was 25 and the doubling time approximately 15 hours, and that no mycoplasma contamination was present and that the rate of spontaneous chromosomal aberrations was less than 5%. The CHL/IU cells were thus recommended for use in experimental investigation of chromosomal aberrations based on a partial revision of the "Experimental Methods Involving New Chemicals".

2.2 Storage

Cryogenic storage in liquid nitrogen.

2.3 Culture media and culture conditions

1) Culture medium

A 10 v/v% neonatal calf serum solution (lot No. NBR10, Mitsubishi Chemical Inc.) was added to Eagle MEM medium (lot No. 431011, Nissui Pharmaceuticals Inc.).

2) Culture conditions

The cells were cultured in an atmosphere with a 5% carbon dioxide concentration at a temperature of 37±0.5°C and a humidity of 100%.

2.4 Cell Line

Cell line subcultures were performed twice a week in dishes with a diameter of 90 mm (made by Nunk). For the experiments cells with a cell line number within 35 were used.

3. S9 mix

3.1 Rat liver S9

S9 (lot No. 00122210, prepared on December 22, 2000, Oriental Yeast Industries Inc.) prepared from the liver of rats (body weight: 207±9.0 g) receiving phenobarbital and 5,6-benzoflavone was used. Until immediately prior to the application of S9, the material was kept in a deep freezer (MDF-U481ATR, Sanyo Electric Inc.) at -80°C.

3.2 Composition of the S9 mix

The S9 mix was prepared so that 1 ml of it contained, at the time of use, 0.3 ml of S9, 5 μmol of $MgCl_2$, 33 μmol of KCl, 5 μmol of glucose-6-phosphate, 4 μmol of NADP and 4 μmol of HEPES (pH 7.2).

4. Cell preculturing

Five ml of cell suspensions containing 1.5 x 10^{-4} cells/ml, or 5 x 10^{-3} cells/ml were cultured for 2 to 3 days in dishes (Iwaki Glass Inc.) with a diameter of 60 mm.

5. Preparation of the test substance and the positive controls

5.1 Preparation of the test substance

1) Acetone (lot No. ELK6246, purity 99.5%, special reagent grade, Wako Pure Pharmaceuticals Inc.) was dehydrated using 5A 1/8 molecular sieves (lot No. CKN3668, Wako Pure Pharmaceuticals Inc.).

2) Reasons for the selection of the solvent

Since the test substance is decomposable by hydrolysis, it is insoluble in a dehydrated DMSO solution at 50 mg/ml, but soluble to more than 276 mg/ml in dehydrated acetone. Moreover, since a 276 mg/ml solution prepared with dehydrated acetone did not show any discoloration or heat development after two hours of its preparation, it could be

judged stable. This is the reason why dehydrated acetone was chosen as solvent.

3) Preparation

The test substance was dissolved in dehydrated acetone. Subsequently this solution was diluted with the solvent to obtain the specified concentration.

4) Preparation timing

The material was kept after preparation at room temperature and used within 2 hours.

5.2 Preparation of positive controls

1) Preparation and storage

MMC and CPA were each dissolved in distilled water, adjusted to concentrations of 0.01 mg/ml and 1 mg/ml and then deep-frozen at -80°C.

2) Preparation timing and storage period

The substance was thawed for application and used within two hours. Deep frozen solutions were used within 6 months after preparation.

6. Short-time treatment

6.1 Cell proliferation inhibition tests

1) Experimental methods

The cells were treated for six hours each in 3 ml of a 30 µl of the prepared test substance solution, or solvent containing culture medium in the absence of S9 mix, or else 2.5 ml of a 30 µl of the prepared test substance solution or solvent in the presence of S9 mix, with 0.5 ml of S9 mix added to 3 ml of the culture medium. The medium was removed at the end of the culture and the cells washed three times with 2 ml of a physiological phosphate buffer solution. Later the material was added to 5 ml of a new culture medium and cultivated for 18 hours.

A total of 50 µl of a 10 mg/ml corsemide solution was then added within 2 hours. At the beginning and end of the treatment the test substance was observed visually to check whether precipitation of the test substance had occurred. After processing, the cells were detached using 0.25% trypsin. A cellcount was taken using a microcell counter (model CDA-500 manufactured by Sysmex Inc.). The cell count in the negative controls was defined as 100% to calculate the cell proliferation rate and later determine the 50% inhibition (IC_{50}) value. Furthermore , 3 ml of 0.075 M KCl were added to the cells separated by centrifugation for 5 minutes at 1,000 rpm and then subjected to a hypotonic treatment for 15 minutes at 37°C. The hypotonically treated cells were then fixed in Carnoy's fluid (methanol-acetic acid=3:1). After dropping the cells onto a slide glass they were stained with 2% Giemsa. One specimen was prepared for each dose.

2) Dose gradation

For the experiments in the absence of S9 mix the maximum dose of 10 mM that had been used, corresponding to 2,760 μg/ml, was clearly cytotoxic. Thus, the maximum dose was set to 46 μg/ml and the dosage divided into 9 grades down to 14 μg/ml. Two dishes were used for each dose.

3) Observation, measurement

During the examination of the mitotic cells, 50 cells in metaphase were observed to determine the incidence of chromosomal aberrations.

(1) Structural aberrations

Structural cell aberrations, excluding gaps, were recorded. Gaps were defined as non-chromosomal areas narrower than chromatids.

(2) Numerical anomalies

The number of cells with a ploidy level in excess of triploidy was recorded.

6.2 Chromosomal aberration experiments

1) Experimental methods

These experiments were performed according to the same experimental methods as that used for the cell proliferation inhibition experiments.

Based on the background data from the Hida factory, positive controls were defined as 0.1 μg/ml of MMC in the absence of S9 mix and 6 μg/ml of CPA in the presence of S9 mix. For each dose 4 specimens were prepared.

2) Dose gradation

The maximum dose, based on the results of the cell proliferation inhibition experiments and the observation of the metaphase division images required for an analysis of chromosomal aberrations, was 34 μg/ml in the absence of S9 mix and the upper limit in the presence of S9 mix 10 mM, corresponding to 2,760 μg/ml.

Thus, the maximum dose in the absence of S9 mix was 34 μg/ml. Taking the cell proliferation rate into account, this was diluted at differential steps of 6 to obtain concentrations of 28 and 22 μg/ml, respectively. In the presence of S9 mix, the maximum dose of 2,760 μg/ml was diluted at differential factors of 2to obtain concentrations of 1,380 and 690 μg/ml, respectively.

3) Observation, measurement

All parameters were coded and the observations performed blinded.

(1) Structural aberrations

For each dose a number of 200 cells in metaphase with a chromosome number of 25±2 were observed. Simultaneously with the recording of cells with structural anomalies the number of cells was classified according to the type of the structural anomalies.

(2) Numerical anomalies

For each dose a number of 200 cells in metaphase were observed and cells with a ploidy level equal to, or in excess of, triploidy were recorded.

7. Continuous treatment

According to the experimental plan we performed cell proliferation inhibition and chromosomal aberration experiments, but because the incidence of chromosomal aberrations had already been confirmed through the short-time treatment, we did not observe the specimens obtained during the chromosomal aberration experiments. We disregarded the results of the cell proliferation inhibition and chromosomal aberration experiments, because they were not considered necessary.

8. Evaluation criteria

The incidence of cells with structural and numerical anomalies increased bymore than 10%. When it was possible to observe that a dose dependence did exist in the incidence pattern of these anomolies or that a 5% or greater increase was reproducible in both the chromosomal abnormality and the confirmation experiments, the results were considered positive. They were considered negative when this was not the case. For the evaluation, no statistical procedures were applied.. For the positive results the D_{20} value (concentration at which aberrations were observable in 20% of the cells) was calculated.

Factors possibly influencing the reliability of the test results

No environmental factors likely to influence the reliability of the test results were observed.

Experimental results

1. Short-time treatment

1.1 Cell proliferation inhibition experiments

The 50% inhibition concentration in the absence of S9 mix was approximately 29 μg/ml and in the presence of S9 mix more than 2,760 μg/ml.

At 34 μg/ml in the absence of S9 mix, the maximum dose used for chromosomal aberration experiments, a clear inhibition of cell proliferation was recognized, with the cell proliferation rate falling to a little under 50%. In the absence of S9 mix, a minor increase in structural anomalies was observed at a concentration of 30 μg/ml.

1.2 Chromosomal aberration experiments (Table 2 and Figure 2)

1) In the absence of S9 mix

(1) Cell proliferation rate

The cell proliferation rate was for 22, 28 and 34 μg/ml 82.3, 44.1 and 17.1% respectively.

(2) Incidence of structural anomalies

The incidence of structural anomalies at 22, 28 and 34 μg/ml was 2.5, 5.5 and 10% respectively. This incidence was 1.0% for the negative controls and 60.5% for the positive control MMC.

(3) Incidence of numerical anomalies

The incidence of numerical anomalies was, in all groups, less than 5%.

(4) D_{20} value

The D20 concentration value in the case of structural anomalies was calculated as 0.076 μg/ml (S=0.0049).

2) In the presence of S9 mix

(1) Cell proliferation rate

The cell proliferation rate at 690, 1,380 and 2,760 μg/ml was 101.0, 95.0 and 95% respectively.

(2) Incidence of structural anomalies

The incidence of structural anomalies at 690, 1,380 and 2,760 μg/ml was 4.5, 2.5 and 0.5% respectively. This incidence was 1.0% for the negative controls and 26.0% for the positive control CPA.

(3) Incidence of numerical anomalies

The incidence of numerical anomalies was, in all groups, less than 5%.

Discussion and conclusions

No marked differences between incidence of abnormal cells between 2 dishes were observed. In the negative controls the proportion of abnormal cells was less than 5%, while the proportion of cells with anomalies other than gaps in the positive controls was more than 20%, suggesting that the experiments had been conducted appropriately.

During short-time treatment in the absence of S9 mix, BS1701 was found to lead to a dose-dependent increase in the incidence of structural anomalies.

Thus, under the conditions of these experiments BS1701 can be considered to have a potential for inducing chromosomal aberrations.